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Molecular evaluation of the phylogenetic position of *Loxostege ayhanana* Kemal & Koçak, 2017 from East Turkey (Lepidoptera: Crambidae, Pyraustinae)

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Abstract

Very few molecular studies on the phylogeny of the *Loxostege* Hübner, [1825] have been performed because molecular data of this highly crowded group are extremely scarce. In the present study, the mitochondrial cytochrome oxidase I gene of *Loxostege ayhanana* Kemal & Koçak, 2017 was firstly sequenced from East Turkey. The phylogenetic trees constructed using neighbor-joining, maximum likelihood and Bayesian inference methods yielded essentially similar topologies. The genus *Loxostege* was not depicted as a monophyletic clade when evaluated within the Pyraustinae. The molecular characterization and phylogenetic position of *L. ayhanana*, which was described by KEMAL & KOÇAK (2017) as a morphological new species, has been firstly identified and supported by molecular phylogenetic analyses in the present study.
KEY WORDS: Lepidoptera, Crambidae, Pyraustinae, *Loxostege ayhanana*, phylogeny, Turkey.

Evolución molecular de la posición filogenética de *Loxostege ayhanana* Kemal & Koçak, 2017 del este de Turquía (Lepidoptera: Crambidae, Pyraustinae)

Resumen

Pocos los estudios moleculares sobre la filogenia de *Loxostege* Hübner, [1825] han sido realizados porque los datos moleculares de este grupo altamente confusos son sumamente escasos. En el estudio actual, el gen mitocondrial citocromo oxidasa I (COI) de *Loxostege ayhanana* Kemal & Koçak, 2017 fue ordenado en serie, en primer lugar, del este de Turquía. El árbol filogenético construido usando el método "neighbor-joining" asociando la probabilidad máxima y la inferencia Bayesiana, produjeron topologías esencialmente similares. El género *Loxostege* no describe un clado monofilético cuando lo valoramos dentro de los Pyraustinae. La caracterización molecular y la posición filogenética de *L. ayhanana*, fue descrita por KEMAL & KOÇAK (2017) como una nueva clase morfológica, en primer lugar, ha sido identificado y sostenido por los análisis de filogenéticos moleculares en el estudio actual.
PALABRAS CLAVE: Lepidoptera, Crambidae, Pyraustinae, *Loxostege ayhanana*, filogenia, Turquía.

Introduction

Loxostege Hübner, [1825] is one of the largest genera in Pyraustinae and shows the most diverse life history adaptations. For *Loxostege*, more than 85 species have been described by traditional morphological methods worldwide (SCHOLTENS & SOLIS, 2015; TRANKNER *et al.*, 2009). In recent years, using traditional morphological methods as well as molecular techniques has become one of the most important developments in Lepidoptera taxonomy and systematics (CHEN *et al.*,

2019; HAUSMANN *et al.*, 2011; SERAPHIM *et al.*, 2018; ZOU *et al.*, 2016). According to other molecular markers, the mitochondrial cytochrome oxidase subunit 1 (COI) gene is the most preferred marker in taxonomy, classification and revision of Lepidoptera. (BUCHNER *et al.*, 2018; KEMAL *et al.*, 2018). Since the mtCOI gene has been used in Lepidoptera molecular systematics, it has been controversial. Because the COI gene is rapidly evolving and it is thought that it cannot fully reflect the phylogeny due to the possibility of high homoplasy due to the rapid saturation of the third codon positions (SOUZA *et al.*, 2016). In contrast, there are numerous studies supporting large-scale datasets that most of the phylogenetic signals are in the third codon position. In addition, the mtCOI gene has significant potential to identify a single species and characterize species boundaries. The variation rate among species is low but the different nucleotide substitution ratios show a higher correlation in determining genetic distances than in other genes (KÄLERSJÖ *et al.*, 1999; RACH *et al.*, 2017).

A better understanding of *Loxostege* phylogeny requires an expansion of the taxon and genome samplings from different geographical locations. In recent years, complete or nearly complete mtCOI gene (658bp) from some *Loxostege* species which are obtained only North America, west Europa and China have been sequenced (CHEN *et al.*, 2019). Since they are serious economic pest of both crops and weeds worldwide, the molecular aspects of *Loxostege sticticalis* (Linnaeus, 1761), *L. cereralis* (Zeller, 1872), *L. allectalis* (Grote, 1877) and *L. commixtalis* (Walker, 1866) within the genus have been studied. However, the phylogenetic relationship of *Loxostege* based on mtCOI sequences has not been discussed comprehensively. Although it is represented by 10 species (*Loxostege aeruginalis* (Hübner, 1796), *L. bicoloralis* (Warren, 1892), *L. clathralis* (Hübner, [1813]), *L. mucosalis* (Herrich-Schäffer, 1848), *L. peltalis* (Eversmann, 1842), *L. peltaloides* (Rebel, 1932), *L. sticticalis* (Linnaeus, 1761), *L. turbidalis* (Treitschke, 1829), *L. wagneri* (Zerny, 1929) and *L. ayhanana* Kemal & Koçak, 2017) (KEMAL & KOÇAK, 2017) in Turkey, there have been almost no significant information regarding molecular of *Loxostege*.

The molecular barcoding of *L. ayhanana* from East Turkey was presented for first time in this study, while the phylogenetic relationships of this species with other species in the genus *Loxostege* were elucidated based on their COI gene. Here, the phylogenetic tree was created at the genera in the subfamily Pyraustinae and the relations of these genera at the molecular taxonomy level were evaluated.

Methods

Loxostege ayhanana specimen (paratype/Lep-Pyr022) was used from Centre for Entomological Studies Ankara (Cesa) Collection for this study (Fig. 1). Total gDNA was extracted from femur part of legs using RED Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA) according to manufacturer instructions and KEMAL *et al.* (2018). The dry legs of individual specimen were washed three times in 100 µL of fresh ethanol (70%). LepF1: ATTCAACCAATCATAAAGATATTGG and LepR1: TAAACTTCTGGATGTCCAAAAAATCA primers (HEBERT *et al.*, 2004) were used for the PCR amplification of mtDNA COI gene. Cycling parameters for PCR amplifications were as follows: Initial denaturation at 94ºC for 2 min, 5 cycles of 94ºC for 30 sec, annealing at 45ºC for 40 sec, and extension at 72ºC for 1 min, 35 cycles of 94ºC for 30 sec, annealing at 51ºC for 40 sec, and extension at 72ºC for 1 min, final extension at 72ºC for 10 min. PCR products were electrophoresed in 1% TAE agarose gels, stained with GelRed, and visualized under UV light. The PCR products were sequenced bi-directionally Macrogen (Netherlands) with LepF1 and LepR1 primers in order to decrease the occurrence of sequencing error by commercial companies. Obtained the sequences were aligned by CodonCode Aligner Programs and their quality was checked and the sequence has been deposited in GenBank (https://www.ncbi.nlm.nih.gov/) with accession number MK883478. Other additional sequences were downloaded from the NCBI/GenBank database and Boldsystem (http://www.boldsystems.org/index.php). Multiple sequence alignments were performed with the ClustalW algorithm implemented in MEGA 7.0 software (TAMURA *et al.*, 2013).

Fig. 1.– *L. ayhanana* Kemal & Koçak, 2017, from Van Province, Bahçesaray, Upper Mukus Valley 1840 m , 23-VI-2016, M. Kemal leg (paratype) used for DNA barcoding.

A total of 160 taxa were employed for phylogenetic analysis, including used *Udea ferrugalis* (Hübner, 1796), *Mecyna asinalis* (Hübner, [1819]) and *Metasia carnealis* (Treitschke, 1829) as outgroup taxa. Sequence divergences between selected sequences were calculated using the Kimura 2-Parameter distance model (KIMURA, 1980) and neighbour-joining (NJ) tree was constructed in the program MEGA 7. Maximum-likelihood (ML) bootstrapping analyses were carried out with 1000 replicates using RA × ML Blackbox with the settings described by STAMATAKIS *et al.* (2008). ML analyses were conducted online using the CIPRES Portal v.3.3 (http://www.phylo.org/). A Bayesian inference (BI) analysis was performed using MrBayes 3.2.6 (RONQUIST & HUELSENBECK, 2003) using the Markov chain Monte Carlo algorithm. The program JModeltest v.2.1.7 (POSADA, 2008) selected the F81 evolutionary model as the best model according to the akaike information criterion for Bayesian inference. The program was run for 10,000,000 generations, with a sample frequency of 100 and a burn-in of 25000.

Results

The existing mtCOI (658bp) DNA barcodes of 27 genera in the subfamily Pyraustinae were used in phylogenetic analyses. ML, NJ, and BI analyses generated similar tree topologies, and three supported values on the NJ tree were shown in Figure 2. The molecular phylogenetic relationships show that the *Loxostege* comprises five main groups: Group A was contained fourteen species of *Loxostege*. In the Group B was only located *Loxostege albiceralis* (Grote, 1878) and had as sister positions with *Arenochroa flavalis* (Fernald, 1894) and *Xanthostege plana* (Grote, 1883) in same clade. Group C, which including *L. ayhanana*, is a sister to this clade.

The base frequencies were A = 31.6%, C = 14.4%, G = 14.9%, and T = 39.1% for the presented

specimen. The sequence character analysis indicated that the GC frequency (29.3%) was apparently lower than the AT frequency (70.7%), which is consistent with the features of the mitochondrial genome for Lepidoptera.

In our phylogenetic tree, it was found that *L. ayhanana* is more closely related to five species than the other species of the genus even though three support values are low. As the NJ tree shows, *L. aeruginalis* (Hübner, 1796) branches as sister group with the *L. virescalis* (Guenée, 1854)-*L. turbidalis* (Treitschke, 1829)-*L. comptalis* (Freyer, 1848) clade, and *L. deliblatica* (Szent-Iványi & Uhrik-Meszáros, 1942) had a basal position. *L. ayhanana* formed a sister taxon to the branch comprising the above five congeners (Fig. 2). In addition, *L. questoralis* (Barnes & McDunnough, 1914), *L. immerens* (Harvey, 1875) and *L. frustalis* (Zeller, 1852) were showed as a sister group with the presented taxon and were located in Group C. *L. nudalis* in the group D was located within the clade consisted from the *Achrya* species. Similarly, *L. sticticalis* (Linnaeus, 1761) in the Group E had a sister position with the populations of *Perispasta caeculalis* (Zeller, 1875), *Pagyda sounanalis* (Legrand, 1966) and *Hahncappsia mellinialis* (Druce, 1899).

The genetic distances between these species with *L. ayhanana* are as follows: *L. ayhanana* and *L. virescalis* (Guenée, 1854) populations have the closest genetic distance 4.83%-5.00%. The second closest was *L. deliblatica* (Szent-Iványi & Uhrik-Meszáros, 1942) with genetic distance 4.99%. It has range 5.17-5.52% with *L. turbidalis* (Treitschke, 1829) populations, and has range 6.00-6.35% with *L. aeruginalis* (Hübner, 1796) populations. *L. ayhanana* has 6.55% genetic distance with *L. comptalis* (Freyer, 1848). The genetic distance between the *L. sticticalis* (Linnaeus, 1761) populations and the *L. ayhanana* was 7.43-7.61%.

Discussion and conclusions

The present work is the first detailed study of the phylogenetic position of the genus *Loxostege* in the subfamily. Although *Loxostege* contains numerous species of this genus in the world, including important species for biological and evolutionary studies as well as many species of economic importance, the diversity and relationships of these species are far from being well understood.

The genus *Loxostege* was not monophyletic taxon according to three support values in molecular phylogenetic analyses and had five distinct lineages (Fig. 2). Although monophyletic groups are generally formed among the different populations of species in presented tree, the monophyly of some species (*L. egregialis* (Munroe, 1976), *L. oberthuralis* (Fernald, 1894) and *L. internationalis* (Munroe, 1976)) should be questioned because of their settlements.

In the numerous studies were reported that only morphological data were unable to give unequivocal answers, but the combined analyses (morphological and molecular analyses) have given a robust phylogenetic estimate for Lepidoptera in recent years (CHEN *et al.*, 2019; MALYSH, 2013; TRANKNER *et al.*, 2009; WAHLBERG *et al.*, 2005). In addition, it is emphasized that morphologically defined species should be supported with molecular analyses for species delimination or phylogenetic position (KEMAL *et al.*, 2019; RAJPOOT *et al.*, 2016). Therefore, for the first time in this study, the molecular evaluation of *L. ayhanana* in the genus was performed using the mtCOI sequence.

One of the factors in favouring the COI gene as an advantageous barcode gene is the apparent distinction power between species in Lepidoptera systematics. In addition, it has characteristic variations in which the distance between species and intraspecies does not coincide. So, the barcoding aperture of COI has been accepted as the most transparent point in the accuracy and reliability of barcode sequences. (MEYER & PAULAY, 2005; RACH *et al.*, 2017). It is a common view for many researchers that a large number of molecular data is needed and that morphological, ecological and molecular synergy will reflect a correct taxonomic and higher level systematic study (KEMAL *et al.*, 2019; RAJPOOT *et al.*, 2016; TRANKNER *et al.*, 2009; WAHLBERG *et al.*, 2005).

The morphologically described *L. ayhanana* species is a typical member of the genus *Loxostege* (KEMAL & KOÇAK, 2017). The monophyly of *L. ayhanana*, which is a distinct species, also supported with the phylogenetic analysis and its genetic distance.

L. nudalis populations in the Boldsystem are in the *Achyra* clade in our phylogenetic tree (Fig. 2).

It is appropriate to change the name of this taxon to *Achrya nudalis* (Hübner, 1796). Also *L. sierralis* was identified by MUNROE (1976) with its four subspecies have been recognized. *L. sierralis internationalis*, *L. sierralis sierralis* and *L. sierralis sanpetealis* have been barcoded (658bp) and *L. sierralis tularealis* has no barcode record available hitherto in Genbank/Boldsystem. The barcode records of these three subspecies in the Boldsystem is given at the species level. In the phylogenetic tree presented in this study, it was shown for the first time that these three populations were different from *L. sierralis* at species level. The maximum genetic distance between *L. sierralis* and *L. internationalis* according to the Kimura 2 parameter is 3.70% and between *L. sierralis* and *L. sanpetealis* is 3.30%. There are three independent species.

After a year of the description of *L. ayhanana* from Turkey, another population has been reported from Crimea (SAVCHUK & KAJGORODOVA, 2018). However, in the present study, we did not have the opportunity to compare these two populations in a molecular level because the mtCOI data of Crimean population is not yet available. Clearly, mtCOI gene sequences for more species of *Loxostege* from more geographic locations require in order to elucidate the phylogeny of the genus.

In the submitted phylogenetic tree, the support values are relatively low thus multiple genes based on more extensive sampling along with new sinapomorphic morphological data are recommended for to overcome the low resolution of single gene analysis. In the present study, as there are the only mtCOI gene sequences of these taxa in the databank (Boldsystem and GenBank), phylogeny estimates were evaluated based on a single barcode. The kinds of some genera do not have molecular barcodes yet, while some genera do not have any molecular data of their type species. Most of the data was obtained from Neotropical and European samples. The phylogeny of this very rich subfamily can be enlightened. With numerous new records obtaining from different geographies. The relationships within the taxa are tried to resolve with this study, an initial one for future studies.

In the presented phylogenetic study, includes *L. ayhanana*, as well as the type species of the genus is *L. aeruginalis* (Hübner, 1796) and four valid species (*L. deliblatica*, *L. comptalis*, *L. turbidalis* and *L. virescalis*) in same clade. According to these results, it has been supported that *L. ayhanana*, which was previously defined morphologically new species, was an independent species by differentiating it from other species of *Loxostege* by molecular analyses.

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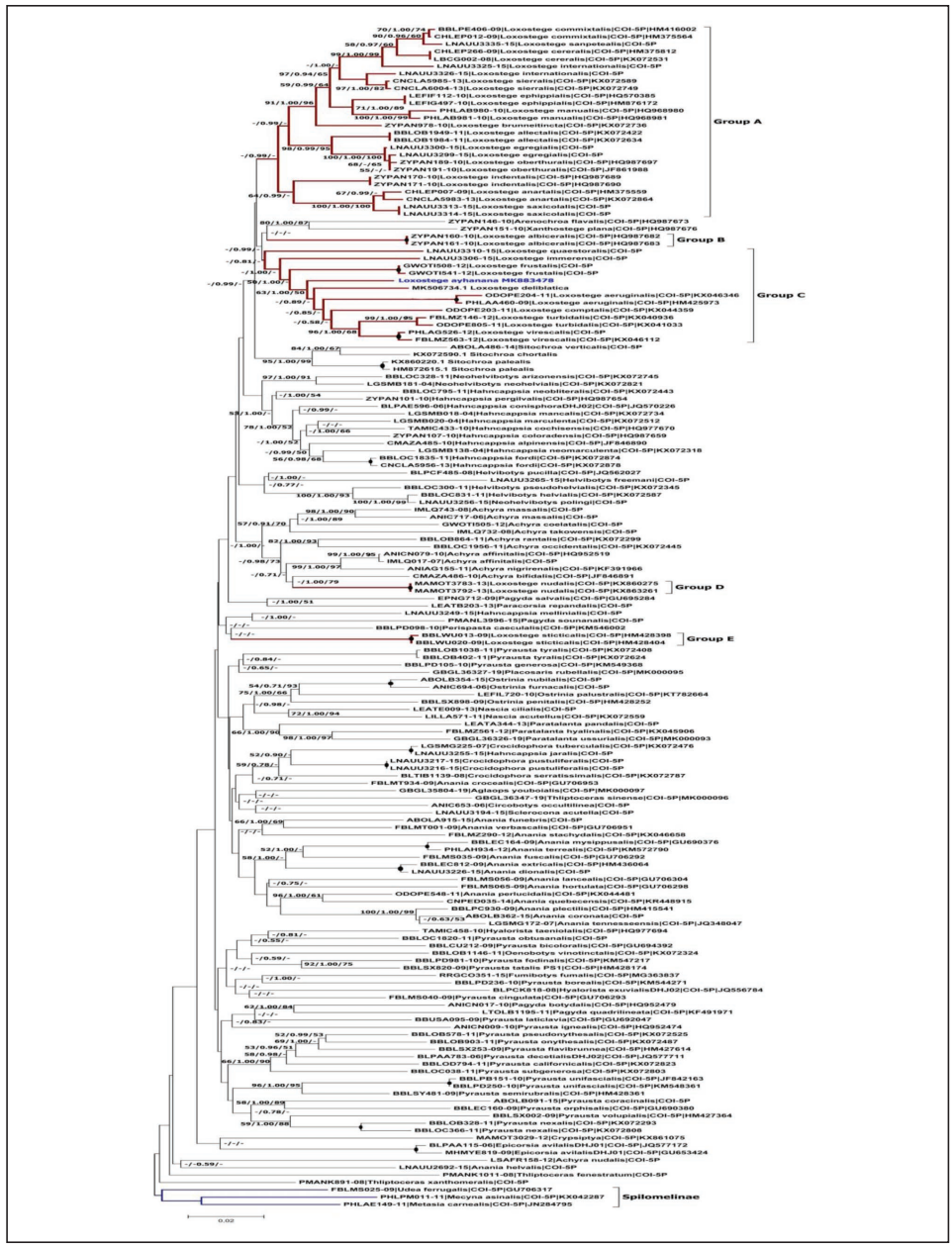

Fig. 2.– NJ, BI and ML analyses based on mtCOI gene sequences of Pyraustinae and some Spilomelinae populations. Numbers at the nodes indicate the NJ bootstrap values, the BI posterior probability and the ML bootstrap values. Fully supported (100/1.00) branches are marked with filled circles. A dash indicates a value less than 0.50 or 50%. Bar, 2 substitutions per 100 nucleotide positions.